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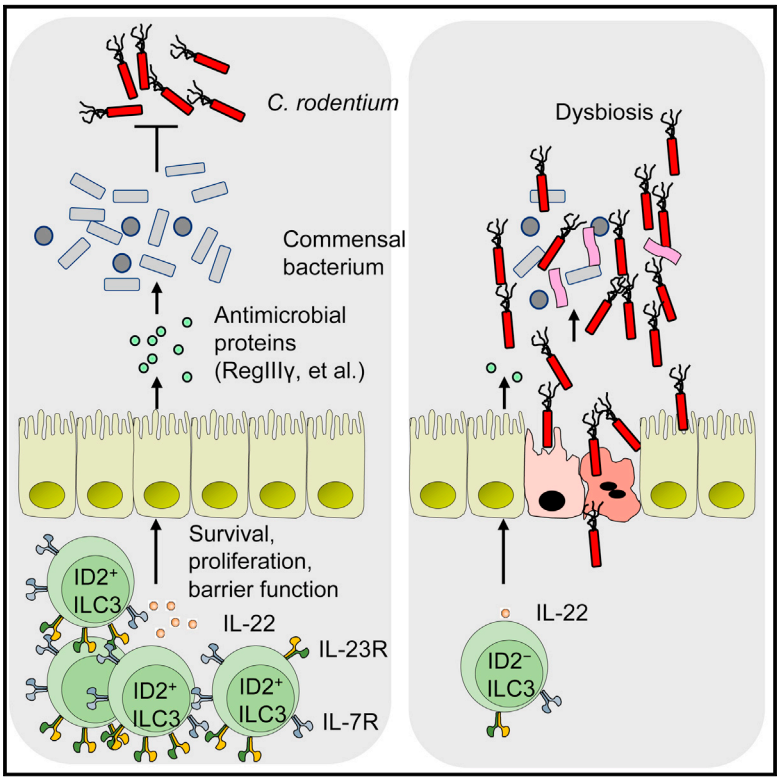
Immunity

Innate Lymphoid Cells Control Early Colonization Resistance against Intestinal Pathogens through ID2-Dependent Regulation of the Microbiota

Graphical Abstract



Highlights

- ID2 is essential to mediate colonization resistance against *C. rodentium*
- Continued expression of ID2 is required for the homeostasis of ILC3s
- ID2-E2A interaction regulates ILC3s producing IL-22 through the AhR and IL-23R pathway
- IL-22 from ILC3s controls colonization resistance through regulating the microbiota

Authors

Xiaohuan Guo, Yong Liang, ..., Barbara L. Kee, Yang-Xin Fu

Correspondence

guoxiaohuan@biomed.tsinghua.edu.cn (X.G.),
yfu@uchicago.edu (Y.-X.F.)

In Brief

Group 3 innate lymphoid cells (ILC3s) are important for protection from gut pathogen infection. Fu and colleagues show that ID2-dependent ILC3s produce IL-22 and limit early colonization of pathogen through homeostasis of the microbiota.

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Innate Lymphoid Cells Control Early Colonization Resistance against Intestinal Pathogens through ID2-Dependent Regulation of the Microbiota

Xiaohuan Guo,[1,2,*] Yong Liang,[1,3] Yuan Zhang,[1] Anna Lasorella,[4] Barbara L. Kee,[1] and Yang-Xin Fu[1,3,*]
[1]Department of Pathology and Committee on Immunology, The University of Chicago, Chicago, IL 60637, USA
[2]Tsinghua University School of Medicine, Beijing 100084, China
[3]Institute of Biophysics and The University of Chicago joint Group for Immunotherapy, Key Laboratory for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, China
[4]Institute for Cancer Genetics, Departments of Neurology and Pathology, Columbia University Medical Center, New York, NY 10032, USA
*Correspondence: guoxiaohuan@biomed.tsinghua.edu.cn (X.G.), yfu@uchicago.edu (Y.-X.F.)
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SUMMARY

Microbiota-mediated effects on the host immune response facilitate colonization resistance against pathogens. However, it is unclear whether and how the host immune response can regulate the microbiota to mediate colonization resistance. ID2, an essential transcriptional regulator for the development of innate lymphoid cell (ILC) progenitors, remains highly expressed in differentiated ILCs with unknown function. Using conditionally deficient mice in which ID2 is deleted from differentiated ILC3s, we observed that these mutant mice exhibited greatly impaired gut colonization resistance against *Citrobacter rodentium*. Utilizing gnotobiotic hosts, we showed that the ID2-dependent early colonization resistance was mediated by interleukin-22 (IL-22) regulation of the microbiota. In addition to regulating development, ID2 maintained homeostasis of ILC3s and controlled IL-22 production through an aryl hydrocarbon receptor (AhR) and IL-23 receptor pathway. Thus, ILC3s can mediate immune surveillance, which constantly maintains a proper microbiota, to facilitate early colonization resistance through an ID2-dependent regulation of IL-22.

INTRODUCTION

Colonization resistance against pathogens is dependent on direct inhibition by the host microbiota (van der Waaij et al., 1971). The microbiota can also regulate the host immune response to mediate indirect colonization resistance against pathogens (Buffie and Pamer, 2013). In contrast, various host genetic factors, including immune factors, also contribute to the varying levels of individual susceptibility to pathogen infections (Chapman and Hill, 2012; Wlodarska et al., 2014). However, it is unclear whether and how such host genetic factors contribute to colonization resistance against pathogens through shaping the microbiota. *Citrobacter rodentium* (*C. rodentium*) is a natural mouse intestinal pathogen that mimics human Enterohaemorrhagic *Escherichia coli* (*E. coli*) and Enteropathogenic *E. coli*, which cause severe diarrhea (Crim et al., 2014; Ochoa et al., 2008). Both innate and adaptive immune components, including interleukin-22 (IL-22)-producing innate lymphoid cells (ILCs), $CD4^+$ T cells, B cells, and *C. rodentium*-specific antibodies, are essential for controlling and eradicating the infection (Bry and Brenner, 2004; Bry et al., 2006; Guo et al., 2014; Zheng et al., 2008). Gut flora has been shown to be important in protection against infection. C3H/HeOuJ mice suffer 100% mortality after *C. rodentium*-induced colitis (Vallance et al., 2003), whereas microbiota transfer from C57BL/6 mice leads to a complete rescue of C3H/HeOuJ mice from death (Ghosh et al., 2011).

IL-22 is induced by IL-23 through the IL-23 receptor (IL-23R)-STAT3 pathway in the early phase of infection and is essential for host defense against *C. rodentium* infection (Guo et al., 2014; Zheng et al., 2008). The major function of IL-22 is to promote mucosal epithelial cell survival and proliferation and to trigger the secretion of antimicrobial peptides, such as RegIIIγ (Pickert et al., 2009). Previous studies have also shown that exogenous RegIIIγ can partially rescue IL-22-deficient mice from death (Zheng et al., 2008). Interestingly, in vitro assays suggest that RegIIIγ can only kill some Gram-positive bacteria, but not the Gram-negative bacteria *C. rodentium* (Cash et al., 2006). Therefore, it is still unknown how IL-22-induced RegIIIγ controls *C. rodentium* infection. Multiple studies have also shown that IL-22 can shape the gut microflora, which contributes to protection or exacerbation of inflammatory bowel disease or infections (Behnsen et al., 2014; Qiu et al., 2013; Zelante et al., 2013). However, it is not known whether IL-22 shapes the microbiota to mediate early colonization resistance against *C. rodentium*.

Group 3 ILCs (ILC3s) are the major producer of IL-22 in the naive gut (Guo et al., 2014; Qiu et al., 2012). ILCs are newly defined immune cells that protect the host from various infections and include group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and $ROR\gamma t^+$ ILC3s (including $CD4^+$ lymphoid tissue-inducer [LTi] cells, natural cytotoxicity receptor $(NCR)^-$ ILC3s, and NCR^+ ILC3s) (Spits et al., 2013). To date, the developmental and functional program of ILC3s is known to involve transcription factors, such as RORγt (Eberl and Littman, 2003; Eberl et al., 2004), AhR (Kiss et al., 2011; Lee et al., 2012; Qiu et al., 2012), and STAT3 (Guo et al., 2014). Recent data suggest that NCR^+

ILC3s (NKp46^{+}RORγt^{+} ILCs) might originate from NCR^{-} ILC3s (Rankin et al., 2013; Vonarbourg et al., 2010). IL-7R signaling is critical for the survival of ILC3s, but it also maintains RORγt expression in mature NCR^{+} ILC3s (Schmutz et al., 2009; Vonarbourg et al., 2010).

E proteins belong to the bHLH transcription factor family, which contains a basic DNA-binding region and a helix-loop-helix (HLH) dimerization domain. They can form homodimers or heterodimers with other HLH proteins and function as transcription activators or repressors. Inhibitor of DNA-binding (ID) proteins are HLH proteins that lack a basic region and can prevent E proteins from binding to DNA. Both E and ID proteins play important roles in lymphoid cell development (Kee, 2009). In particular, ID2 is thought to be required for the development of the ILC precursor given that *Id2*$^{-/-}$ mice lack all the currently known ILCs, including groups 1–3 (Boos et al., 2007; Hoyler et al., 2012). ID2 is continuously and highly expressed in all ILCs, including differentiated ILCs (Hoyler et al., 2012). However, the function of ID2 in these well-differentiated ILCs is still unclear, given that *Id2*$^{-/-}$ mice lack ILCs from the earliest identifiable stage. In this study, we used conditional deletion of *Id2* after RORγt expression in the ILC3 lineage to demonstrate that continuous ID2 expression is required for the homeostasis and function of ILC3s. Using this system, we showed that ILC3s are essential for regulating the microbiota to mediate early colonization resistance against intestinal pathogens.

RESULTS

ID2 Is Continuously Expressed in Intestinal ILCs

To test whether ID2 can function in differentiated ILCs, we first analyzed ID2 expression in different ILC populations with *Id2*$^{GFP/+}$ mice. As previously reported (Cherrier et al., 2012; Hoyler et al., 2012), ID2 was expressed in differentiated ILCs, including natural killer (NK) cells, ILC1s, ILC2s, and ILC3s (Figure S1A). ID2 was not homogeneously expressed in the different subsets of ILC3s. NCR^{+} ILC3s expressed higher concentrations of ID2 than did LTi cells and NCR^{-} ILC3s (Figure S1B). Because NCR^{+} ILC3s could be differentiated from NCR^{-} ILC3s (Rankin et al., 2013; Vonarbourg et al., 2010), our findings raise the possibility that ID2 might continue to play a role in the development and function of ILC3s after their formation.

To study the requirements for ID2 in the homeostasis and function of differentiated ILC3s, we crossed *Id2*$^{fl/fl}$ mice with *Rorc*^{cre} transgenic mice to achieve specific deletion of *Id2* after RORγt expression in ILC3s (*Rorc*^{cre}*Id2*$^{fl/fl}$). Because RORγt is transiently expressed at high levels at the double-positive stage of T cell development, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice lack ID2 expression not only in RORγt^{+} ILC3s but also in most αβ T cells (Figure S1C).

ID2 Is Essential for Early Colonization Resistance and Protection against *C. rodentium* Infection

Previous studies have shown that ILC3s are essential for host protection against *C. rodentium* infection (Guo et al., 2014; Qiu et al., 2012). We next investigated the importance of ID2 for ILC3 function in this infection model. After receiving high doses of *C. rodentium* infection, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice rapidly lost body weight and died around day 10, whereas no weight loss or death was observed in their littermate control *Id2*$^{fl/fl}$ mice (Figures 1A and 1B). Consistent with the increased morbidity and mortality, bacterial titers were 10×–100× higher in the feces of *Rorc*^{cre}*Id2*$^{fl/fl}$ mice than in the feces of *Id2*$^{fl/fl}$ mice at post-infection day 5 (Figure 1C). Systemic dissemination of *C. rodentium* in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice, as evidenced by increased bacterial titers in the blood, liver, and spleen, was also observed (Figures 1D and 1E). The *Rorc*^{cre}*Id2*$^{fl/fl}$ mice also exhibited severe diarrhea, inflammation, and colon pathology upon *C. rodentium* challenge (data not shown and Figure 1F). Collectively, these data demonstrate that continued ID2 expression in RORγt^{+} cells is required for host defense against *C. rodentium* infection.

Because disease signs, including diarrhea and loss of body weight, appeared in ID2-deficient mice before post-infection day 5, we hypothesized that an ID2-dependent mechanism influenced the intestinal environment to limit early colonization even before the innate response was initiated. To test our hypothesis, we challenged both *Rorc*^{cre}*Id2*$^{fl/fl}$ and *Id2*$^{fl/fl}$ littermate mice with a low dose of *C. rodentium* to determine whether *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were more readily colonized. Interestingly, there was significantly more colonization of *C. rodentium* in the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice in the first few days, even as early as post-infection day 1, than in the *Id2*$^{fl/fl}$ mice (Figure 1G). Moreover, *C. rodentium* could be detected in the whole intestine of *Rorc*^{cre}*Id2*$^{fl/fl}$ mice even at post-infection day 1, whereas it was detected only in the cecum of *Id2*$^{fl/fl}$ mice (Figure S1D). Similar to mice infected with a high dose, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice infected with a low dose of *C. rodentium* had severe diarrhea, rapidly lost body weight, and died around day 10, whereas *Id2*$^{fl/fl}$ mice were not affected (Figures 1H and 1I). Together, our data indicate that ID2 is essential in RORγt^{+} cells for host defense against a mucosal bacterial pathogen and is required for maintenance of early colonization resistance.

Given that it takes a few days for an effective innate immune response to control a pathogen, the impaired early colonization resistance against *C. rodentium* suggested that the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice had a pre-existing defect. Previous studies have shown that either increased inflammation or a reduced mucus layer could result in increased colonization of an intestinal pathogen (Bergstrom et al., 2010; Wlodarska et al., 2011). However, colonic mRNA analysis showed that there was no increased expression of pro-inflammatory cytokines or reduction of mucin in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (Figures S1E and S1F). Moreover, H&E and PAS (periodic acid-Schiff) staining showed that there were no obvious alterations of histopathology, mucus layers, or goblet cells (Figure S1G). Together, these data suggest that the increased *C. rodentium* colonization in our ID2-deficient mice was unlikely to be due to changed colonic inflammation or mucus environment.

The ID2-Dependent Microbiota Controls Colonization Resistance against *C. rodentium* Infection

The host microbiota has been recognized as a direct mediator for colonization resistance against pathogens (Buffie and Pamer, 2013). To test whether ID2 regulates early *C. rodentium* colonization through the microbiota, we first treated both *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice with antibiotics for 1 week and then infected these mice with a low dose of *C. rodentium* after 1 day of rest. As shown in Figure 2A, fecal *C. rodentium* titers in control-treated *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were much higher than in *Id2*$^{fl/fl}$ mice at days 1 and 3 after infection. However, bacterial titers

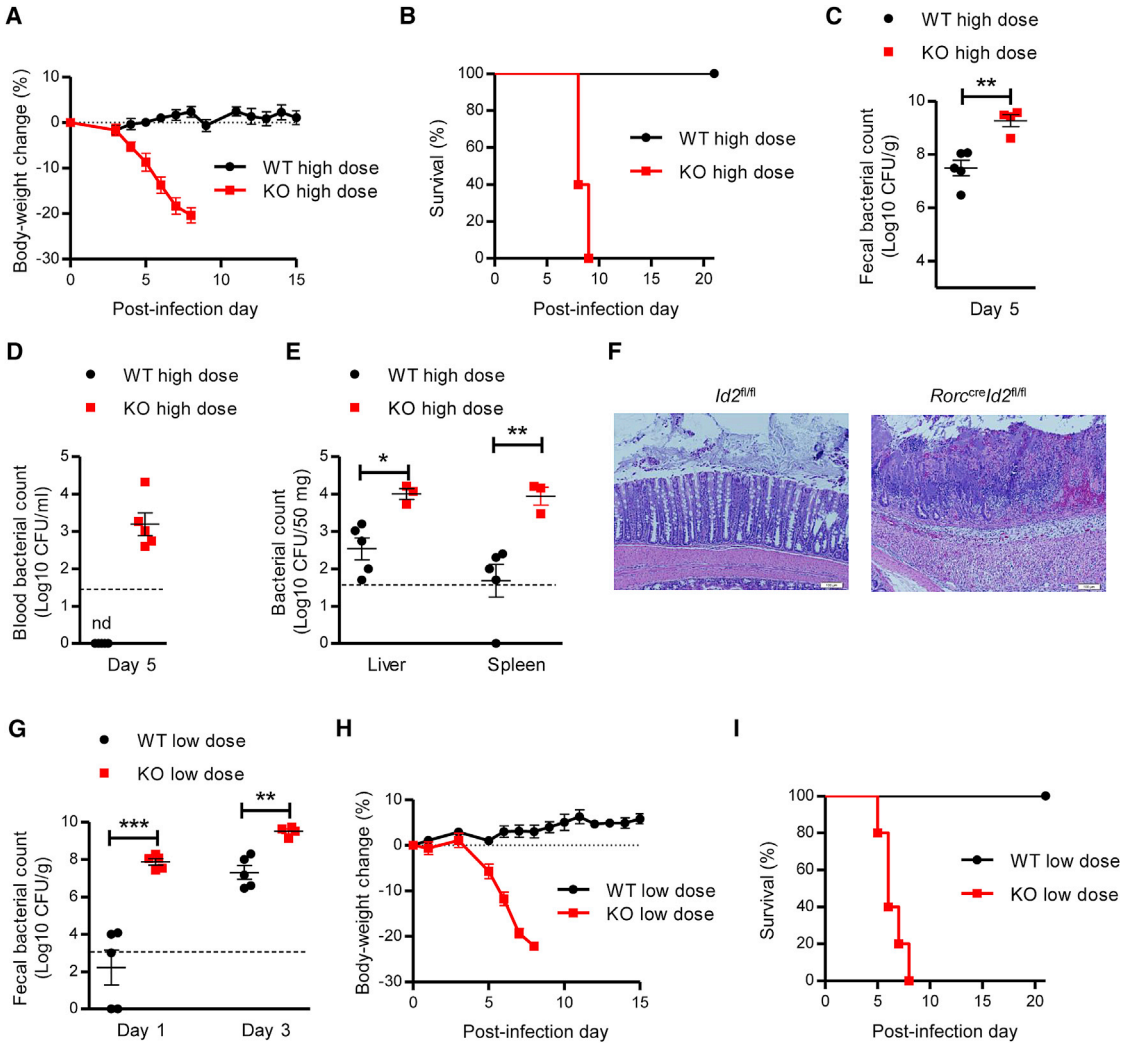

Figure 1. ID2 Is Essential for Mediating Colonization Resistance and Protection against *C. rodentium* Infection

(A–F) 7-week-old *Rorc*^{cre}*Id2*$^{fl/fl}$ (knockout [KO]) mice (n = 5) and their wild-type (WT) *Id2*$^{fl/fl}$ littermates (n = 5) were orally inoculated with a high dose (2 × 10^9 CFU) of *C. rodentium*. Body-weight change (A) and survival rates (B) are shown. Fecal and blood *C. rodentium* titers at post-infection day 5 (C and D, respectively) and *C. rodentium* titers from spleen and liver homogenate cultures at post-infection day 8 (E) are shown. The dashed line indicates the limit of detection. (F) Histological analysis of representative colons from WT and KO mice at post-infection day 8. Scale bars represent 100 μm.

(G–I) 7-week-old KO mice (n = 5) and their WT littermates (n = 5) were orally inoculated with a low dose (5 × 10^6 CFU) of *C. rodentium*. (G) Fecal *C. rodentium* titers at the indicated post-infection day are shown. Body-weight change (H) and survival rates (I) are shown.

Each dot represents one individual mouse (C–E and G). Error bars represent the SEM. *p < 0.05; **p < 0.01; ***p < 0.001 (Student's t test); nd, nondetectable. Data are representative of three (A–D and G–I) or two (E and F) independent experiments. See also Figure S1.

were increased in both the *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice after antibiotic treatment during the first few days after infection (Figure 2A). In addition, both *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ antibiotic-treated mice rapidly lost more body weight than did untreated mice (Figure 2B). However, only a little systemic dissemination of *C. rodentium* was observed in the blood and liver of *Id2*$^{fl/fl}$ antibiotic-treated mice, despite high pathogen titers in the feces (Figures 2A, S2A, and S2B). Together, these results suggest that the microbiota is critical for early colonization resistance against *C. rodentium* but that it has a limited effect on systemic dissemination of the pathogen.

Our data led us to hypothesize that ID2 controls the microbiota to maintain colonization resistance. To test this hypothesis, we reconstituted gnotobiotic C57BL/6 mice with cecal content from *Id2*$^{fl/fl}$ or *Rorc*^{cre}*Id2*$^{fl/fl}$ mice by oral inoculation. One day later, the reconstituted mice were challenged with a low dose of *C. rodentium*. As shown in Figure 2D, germ-free mice that did not receive a microbiota transplantation exhibited very high fecal *C. rodentium* titers even at day 1, whereas mice reconstituted with the *Id2*$^{fl/fl}$ microbiota showed markedly reduced *C. rodentium* titers. Importantly, germ-free mice inoculated with *Rorc*^{cre}*Id2*$^{fl/fl}$ cecal content showed higher *C. rodentium* titers than did *Id2*$^{fl/fl}$ mice (Figure 2D). These data demonstrate that the ID2-dependent microbiota controls colonization resistance against *C. rodentium*.

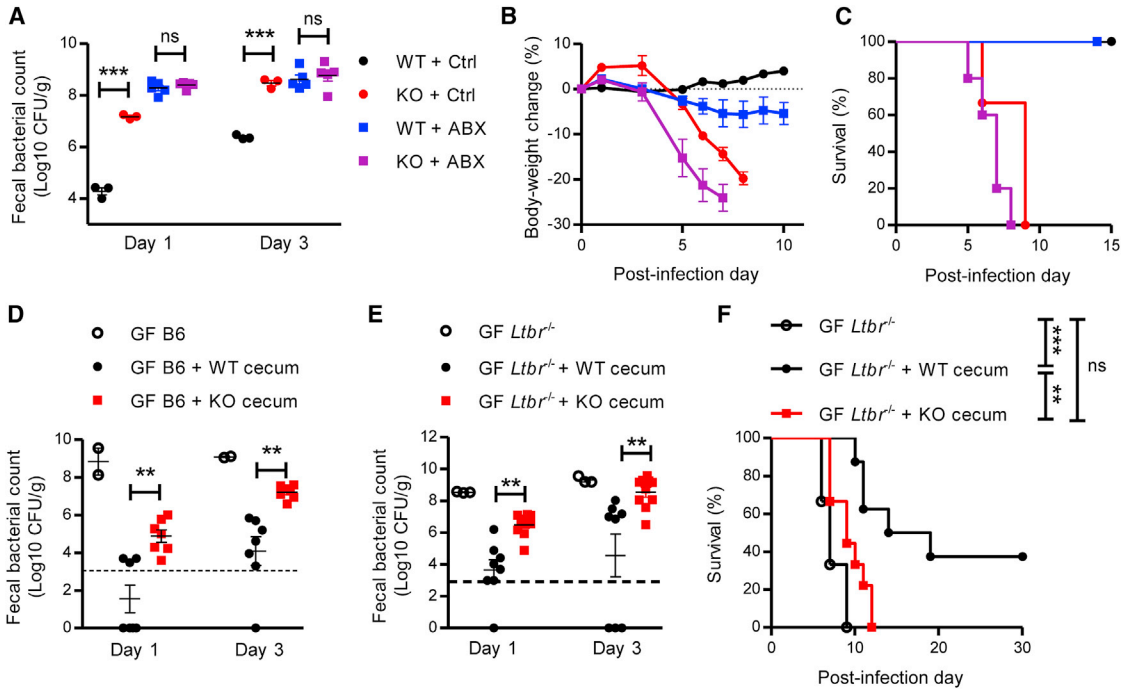

Figure 2. The ID2-Dependent Microbiota Mediates Colonization Resistance against *C. rodentium* Infection

(A–C) 7-week-old *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice and their *Id2*$^{fl/fl}$ (WT) littermates were treated with either antibiotics (n = 5) or a control (n = 3) in drinking water for 1 week. One day later, WT and KO mice were orally inoculated with 5 × 10^6 CFU of *C. rodentium*. Fecal *C. rodentium* titers at the indicated post-infection day (A), body-weight change (B), and survival rates (C) are shown. Data are representative of two independent experiments.

(D–F) Germ-free (GF) WT B6 mice (D) or GF *Ltbr*$^{-/-}$ mice (E and F) were colonized with the microbiota from either WT or KO littermate mice by gavage of cecal material. One day later, these mice were orally inoculated with 5 × 10^6 CFU (D) or 1 × 10^7 CFU (E and F) of *C. rodentium*, and fecal *C. rodentium* titers were examined at the indicated post-infection day (D and E). (F) Survival rates were monitored every day. Data were pooled from three independent experiments. Each dot represents one individual mouse (A, D, and E). The dashed line indicates the limit of detection. Error bars represent the SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference (Student's t test). See also Figure S2.

The microbiota can also indirectly mediate colonization resistance through regulation of host immune response (Buffie and Pamer, 2013). Reconstitution of the microbiota in germ-free mice will induce the host immune response, which might result in increased indirect colonization resistance against *C. rodentium*. We examined whether the different microbiota from *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice induce different host immune responses of germ-free mice. As shown in Figure S2C, reconstitution of *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ microflora in germ-free mice induced similar amounts of expression of pro-inflammatory cytokines and antimicrobial proteins. Together, these data indicate that early colonization resistance mediated by the ID2-dependent microbiota might not be through the regulation of host immune response.

Ltbr$^{-/-}$ mice have multiple defects in innate and adaptive immune responses, including reduced IL-22 and antibody production, which are both essential for host protection against *C. rodentium* infection (Kang et al., 2002; Spahn et al., 2004; Tumanov et al., 2011; Wang et al., 2010). To further determine whether the ID2-dependent microbiota can directly mediate colonization resistance, we used *Ltbr*$^{-/-}$ germ-free mice as recipients. As observed in the C57BL/6 germ-free recipient mice, the *Ltbr*$^{-/-}$ germ-free mice repopulated with the *Id2*$^{fl/fl}$ microbiota showed reduced *C. rodentium* colonization, whereas the *Ltbr*$^{-/-}$ mice repopulated with the *Rorc*^{cre}*Id2*$^{fl/fl}$ microbiota showed more *C. rodentium* colonization (Figure 2E). Moreover, transplantation of the *Id2*$^{fl/fl}$ microbiota improved the survival of *Ltbr*$^{-/-}$ mice after *C. rodentium* challenge, whereas *Ltbr*$^{-/-}$ germ-free mice transferred with *Rorc*^{cre}*Id2*$^{fl/fl}$ cecal content showed a mortality rate similar to that of the untreated *Ltbr*$^{-/-}$ germ-free mice (Figure 2F). All together, these data indicate that the microbiota mediates colonization resistance, which is dependent on continued expression of ID2 in RORγt$^+$ cells.

To further understand how the ID2-dependent microbiota regulates pathogen colonization, we examined the microbiome by pyrosequencing with bacterial 16S rRNA genes. Analysis of 16S rRNA genes revealed that although there were no obvious differences in the bacterial diversity and compositions at the phyla level between *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (Figures S2D and S2E), we observed that several operational taxonomic units were over- and underrepresented in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (Figure S2F). Furthermore, qPCR with primers specific to different bacteria demonstrated that segmented filamentous bacteria (SFBs) were overgrown in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice (Figure S2G); these bacteria have been shown to induce the development of T helper 17 (Th17) cells and be regulated by ILC3s (Ivanov et al., 2009; Qiu et al., 2013). All together, our data suggest that ID2 in RORγt$^+$ cells regulates the intestinal microbiota.

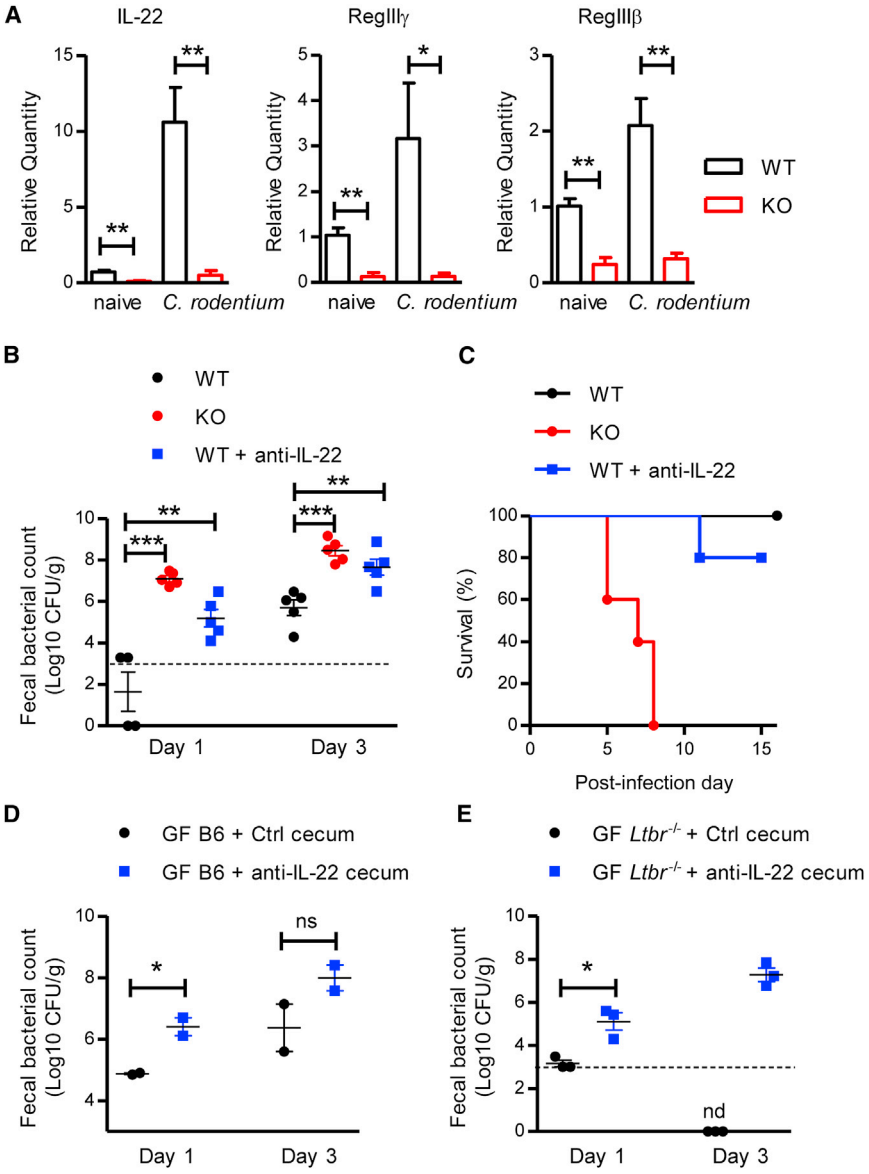

Figure 3. ID2 Mediates Colonization Resistance against *C. rodentium* through IL-22-Dependent Regulation of the Microbiota

(A) *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice and their *Id2*$^{fl/fl}$ (WT) littermates were infected with 2 × 10^9 CFU of *C. rodentium*. The mRNA expression of antimicrobial proteins IL-22, RegIIIγ, and RegIIIβ in the colon of naive mice or mice on day 5 post-infection was measured by real-time PCR. Data are representative of two independent experiments (n = 3–5 per group; mean ± SEM).

(B and C) WT mice were injected intraperitoneally with either anti-IL-22 antibody (8E11.9, 100 μg per mouse per week, n = 5) or mouse IgG control (n = 5) at 3, 4, or 5 weeks old. Two weeks later, 7-week-old WT and KO mice were orally infected with a low dose (5 × 10^6 CFU) of *C. rodentium*. Fecal *C. rodentium* titers at the indicated post-infection day (B) and survival rates (C) are shown. The dashed line indicates the limit of detection. Data are representative of two independent experiments.

(D and E) Germ-free (GF) WT B6 mice (D) or GF *Ltbr*$^{-/-}$ mice (E) were colonized with the microbiota from WT mice treated with either anti-IL-22 or mouse IgG by gavage of cecal material. One day later, these mice were orally inoculated with a low dose (5 × 10^6 CFU in D and 1 × 10^7 CFU in E) of *C. rodentium*, and fecal *C. rodentium* titers were examined at the indicated post-infection day. Data are representative of two (D) or three (E) independent experiments.

Each dot represents one individual mouse (B, D, and E). Error bars represent the SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ns, no significant difference (Student's t test); nd, nondetectable. See also Figure S3.

ID2 Mediates Colonization Resistance against *C. rodentium* through IL-22-Dependent Regulation of the Microbiota

IL-22, mainly produced by RORγt$^+$ cells, not only is required for protection against pathogen infection but also regulates the homeostasis of microflora in the intestine (Qiu et al., 2013; Qiu et al., 2012). We tested whether ID2 mediates colonization resistance through IL-22-dependent regulation of the microbiota. *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were infected with *C. rodentium*, and the expression of *Il22* mRNA and mRNA encoding two IL-22-dependent antimicrobial proteins (RegIIIγ and RegIIIβ) was examined in both naive and infected colon tissues. As shown in Figure 3A, compared with *Id2*$^{fl/fl}$ mice, *Rorc*^{cre}*Id2*$^{fl/fl}$ mice showed significant reduction of *Il22*, *Reg3g*, and *Reg3b* mRNA in both naive and infected states, indicating that continued ID2 expression in RORγt$^+$ cells is essential for IL-22 production in the intestine. To test whether IL-22 regulates the microbiota to mediate early colonization resistance, we treated *Id2*$^{fl/fl}$ mice with anti-IL-22 neutralization antibody at weaning and challenged them with *C. rodentium* when they were 7 weeks old. Similar to the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice, *Id2*$^{fl/fl}$ mice treated with anti-IL-22 showed increased fecal *C. rodentium* amounts and decreased survival (Figures 3B and 3C), indicating that blocking IL-22 function early in life destroys colonization resistance in adulthood. To further determine whether early colonization resistance is mediated by the IL-22-dependent microbiota, but not the *C. rodentium*-induced immune response, we treated *Id2*$^{fl/fl}$ mice with anti-IL-22 antibody 7 days before or at the same day of infection. As shown in Figures S3A–S3C, although only early anti-IL-22 treatment resulted in increased colonization of *C. rodentium* in feces, both anti-IL-22-treated groups revealed increased *C. rodentium* colony-forming units (CFU) in blood, spleen, and liver. Our earlier data in this study showed that the antibiotic-treated microbiota dramatically reduced early colonization resistance against *C. rodentium*, but not systemic dissemination (Figures S3A and S3B). Thus, our data indicate that IL-22 not only mediates colonization resistance against intestinal pathogen through regulation of microbiota but also controls the systemic dissemination

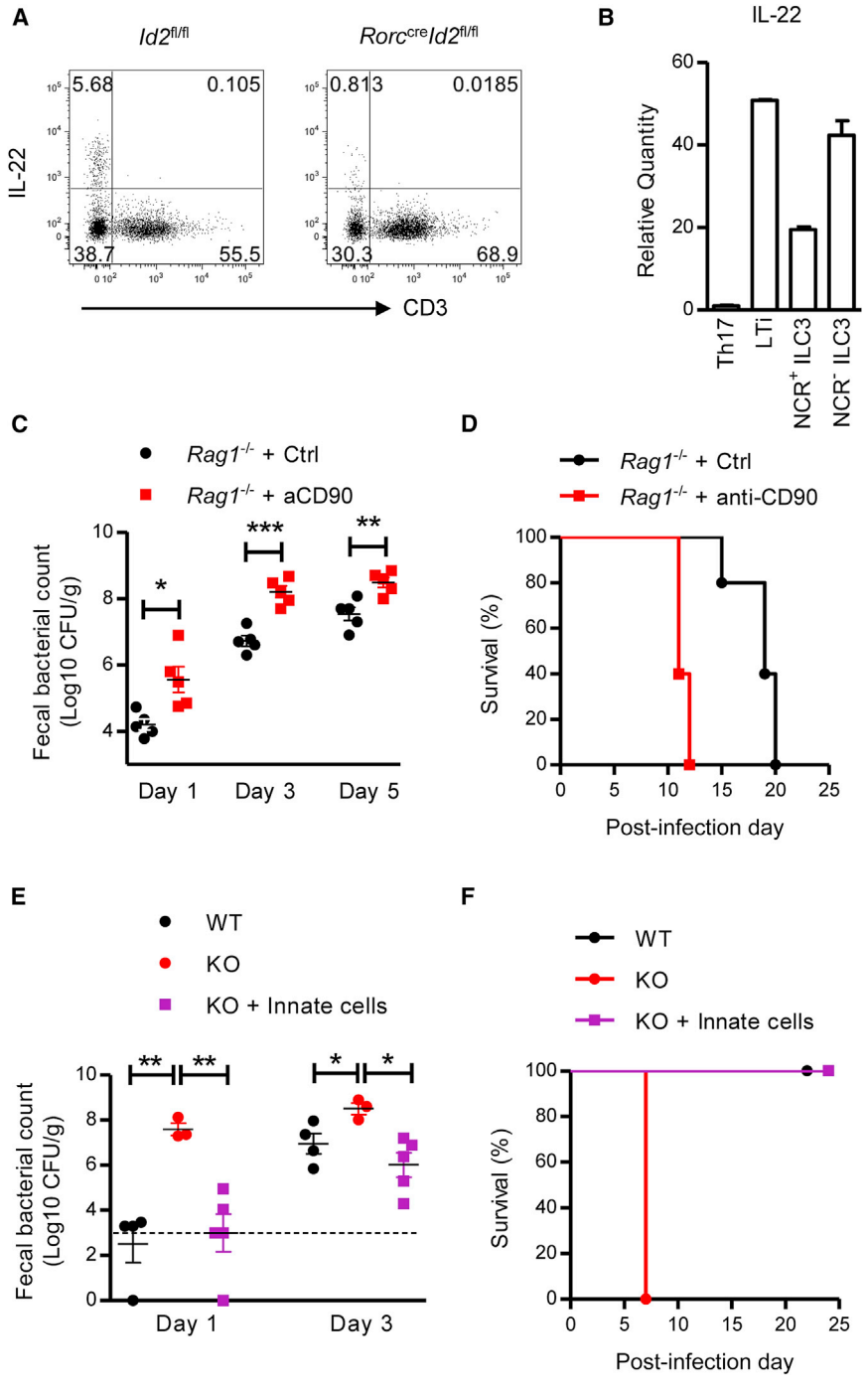

Figure 4. IL-22-Producing ILCs Mediate Colonization Resistance against *C. rodentium*

(A) IL-22 expression in $CD3^-$ and $CD3^+$ cells was analyzed by intracellular cytokine staining. Intestinal LPLs were isolated from the colons of naive *Id2*$^{fl/fl}$ (WT) or *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice, stimulated with IL-23 (25 ng/ml) for 4 hr, and gated in $Thy1^+$ lymphocytes. Data are representative of at least five independent experiments.

(B) $ROR\gamma t^+$ ILC3s and T helper cells were purified by flow cytometric sorting from intestinal LPLs of *Rorc*$^{GFP/+}$ mice. The mRNA expression of IL-22 was measured by real-time PCR. Data are representative of two independent experiments (mean ± SEM of triplicate samples of real-time PCR).

(C and D) *Rag1*$^{-/-}$ mice were injected intraperitoneally with either anti-CD90 antibody (30H12, 100 μg per mouse each time) or rat IgG control at days −10 and −5 before infection for depletion of ILCs (n = 5). Ten days later, mice were orally infected with 1×10^7 CFU of *C. rodentium*. Fecal *C. rodentium* titers at the indicated post-infection day (C) and survival rates (D) are shown. Data are representative of two independent experiments. Each dot represents one individual mouse (C).

(E and F) KO mice were injected with innate cells at weaning. Four weeks later, WT and KO mice were orally infected with 5×10^6 CFU of *C. rodentium*. Fecal *C. rodentium* titers at the indicated post-infection day (E) and survival rates (F) are shown. The dashed line indicates the limit of detection. Data are representative of two independent experiments.

Error bars represent the SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Student's t test). See also Figure S4.

of pathogen, which is less microbiota dependent. Consistent with this, *Rorc*^{cre}*Stat3*$^{fl/fl}$ mice, which lack IL-22 production from $ROR\gamma t^+$ cells, also exhibited impaired early colonization resistance (Figure S3D). To further test the requirement for IL-22 to maintain a protective microbiota, we transplanted germ-free C57BL/6 and *Ltbr*$^{-/-}$ mice with microflora from the anti-IL-22-treated or control mice and challenged them with *C. rodentium*. As observed with germ-free mice repopulated with the microbiota from *Rorc*^{cre}*Id2*$^{fl/fl}$ mice, the microbiota from anti-IL-22-treated mice failed to prevent colonization by *C. rodentium* (Figures 3D and 3E). Collectively, our results suggest that ID2 mediates colonization resistance against *C. rodentium* through IL-22-dependent regulation of the microbiota.

IL-22-Producing ILCs Are Necessary and Sufficient to Mediate Colonization Resistance against *C. rodentium*

Although IL-22 can be produced by both ILC3s and T cells, we observed that ILC3s were the main producer of IL-22 in the naive state (Figures 4A and 4B) and that innate IL-22 production was markedly reduced in ID2-deficient mice (Figure 4A). This IL-22 expression profile suggests that ILC3s might be responsible for colonization resistance. To test our hypothesis, we depleted ILCs with anti-CD90 antibody in *Rag1*$^{-/-}$ mice 1 week before infection. Compared with control mice, ILC-depleted *Rag1*$^{-/-}$ mice showed increased early *C. rodentium* colonization (Figure 4C) and a decreased lifespan after infection (Figure 4D). Furthermore, innate cells from the intestine of *Rag1*$^{-/-}$ mice were isolated and transferred into *Rorc*^{cre}*Id2*$^{fl/fl}$ mice at weaning. Consistent with our hypothesis,

innate cells restored the expression of IL-22 and colonization resistance against *C. rodentium* in *Rorc*^{cre}*Id2*$^{fl/fl}$ mice and completely rescued the *Rorc*^{cre}*Id2*$^{fl/fl}$ mice from death (Figures 4E, 4F, and S4A). Together, these results demonstrate that IL-22-producing ILCs mediate early colonization resistance against an intestinal pathogen.

Continued Expression of ID2 Is Required for ILC3 Development and Maintenance

Because ID2 expression in ILC3s is essential for IL-22-dependent regulation of the microbiota against pathogen colonization, we next determined how ID2 regulates IL-22 production by ILC3s and whether ID2 regulates ILC3 homeostasis or functionality. ID2 is required for the development of all ILC precursors; thus, first we tested whether ID2 is still important for further development of ILC3s. *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ mice were crossed with *Rorc*$^{GFP/+}$ mice, and ILC3s were examined in the intestinal lamina propria leukocytes (LPLs). RORγt-expressing ILC3s were dramatically reduced in both the large and small intestines in *Rorc*$^{GFP/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice, and the remaining ILC3s were mostly CD4$^+$ LTi cells and NCR$^-$ ILC3s (Figures 5A–5C, S5A, and S5B). Previous studies have shown that NCR$^+$ ILC3s can develop from NCR$^-$ ILC3s and that IL-7R signaling is required for this further development, as well as for the survival and proliferation of ILCs (Schmutz et al., 2009; Vonarbourg et al., 2010). We found that ID2-deficient ILC3s had diminished IL-7Rα expression at both the protein and mRNA levels (Figures 5D and 5E).

To further determine the role of ID2 in ILC3 maintenance, we sorted both wild-type (WT) and *Rorc*^{cre}*Id2*$^{fl/fl}$ ILC3s from CD45.1$^+$ C57BL/6 and CD45.2$^+$ *Rorc*^{cre}*Id2*$^{fl/fl}$ mice and injected them into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice at a 1:1 ratio. The gut LPLs were isolated 4 weeks later, and flow cytometric analysis revealed that WT ILC3s were 4-fold more abundant than *Rorc*^{cre}*Id2*$^{fl/fl}$ cells (Figure S5C). Moreover, we found that ID2-deficient ILC3s exhibited increased apoptosis and reduced Bcl-2 expression, which is considered an important anti-apoptotic protein (data not shown and Figure S5D). Given that ID2 is required for the development of all ILCs and that early developmental defects might also result in dramatic reduction of adult ILC3s (Cherrier et al., 2012), we further isolated the fetal liver cells to examine whether ID2 is required for the development rather than the maintenance of ILC3s. As shown in Figures 5F and 5G, there was no reduction of fetal LTi cells in the liver of *Rorc*^{cre}*Id2*$^{fl/fl}$ mice. However, similar to adult ILC3s, ID2-deficient fetal ILC3s also exhibited reduced expression of IL-7Rα (Figures 5H and 5I). Together, our results clearly demonstrate that ID2 expression continues to be required in ILC3s after expression of RORγt for proper expression of IL-7Rα and ILC3 maintenance.

ID2 Regulates IL-22 Production by ILC3s through the IL-23R Pathway

Multiple transcription factors, such as RORγt, AhR, and GATA-3, regulate ILC3 development and function (Qiu et al., 2012; Serafini et al., 2014). To further test whether ID2 also regulates the function of ILC3s, we isolated both *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ LPLs from the intestine and examined IL-22 production. Interestingly, the residual ID2-deficient ILC3s were unable to produce IL-22 after IL-23 stimulation (Figures 6A and 6B). This was also confirmed by the observation that IL-22 production in LTi cells and NCR$^-$ ILC3s sorted from *Rorc*$^{GFP/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice was markedly lower than that in *Rorc*$^{GFP/+}$*Id2*$^{fl/fl}$ ILC3s (Figures 6C and S6A). Together, these data suggest that ID2 also plays an essential role in regulating the function of ILC3s.

Previous studies have shown that IL-23 can interact with IL-23R and activate STAT3. RORγt, AhR, and STAT3 can bind to *Il22* and directly promote IL-22 production (Guo et al., 2014; Qiu et al., 2012). To determine how ID2 regulates ILC3s producing IL-22, we first examined the expression of these transcription factors and cytokine receptor. LTi cells and NCR$^-$ ILC3s were purified from both *Rorc*$^{GFP/+}$ and *Rorc*$^{GFP/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice. Consistent with a previous study (Zhang et al., 2014), ID2-deficient LTi cells showed increased RORγt expression. The expression of AhR was reduced in both ID2-deficient resting LTi cells and NCR$^-$ ILC3s, but not IL-23-activated LTi cells. Interestingly, the expression of IL-23R and STAT3 was reduced in both ID2-deficient LTi cells and NCR$^-$ ILC3s (Figures 6D and S6A). Moreover, ID2-deficient fetal liver LTi cells also showed significant reduction of IL-22 and IL-23R (Figure S6B), suggesting that early ID2 regulation of IL-22 might be through the IL-23R-STAT3 pathway.

ID proteins are transcription factors that inhibit the function of the E protein transcription factors by preventing them from binding to DNA. Previous studies have shown that ID2 regulates the development of NK and LTi cells through suppression of E2A, also known as TCF3 (Boos et al., 2007). To test whether ID2 also regulates IL-22 production through suppression of E2A, we infected EL4 cells with retrovirus expressing either ID2 or E47 (one E protein encoded by *E2A*, also known as *Tcf3*) and examined IL-22 production. Because RORγt is the master regulator for IL-22 production, we also infected the EL4 cells with or without RORγt-expressing retrovirus. As shown in Figures 6E and 6F, compared to the control group (with empty MigR retrovirus), EL4 cells infected with ID2 retrovirus produced more IL-22, whereas the E47 group showed reduced IL-22 production with or without RORγt expression. Moreover, increased expression of ID2 also caused increased IL-23R and STAT3 expression in EL4 cells, whereas overexpression of E47 inhibited IL-23R expression. Together, our data indicate that E2A might regulate IL-22 production through suppression of the IL-23R pathway and that ID2 promotes IL-22 production through suppression of E2A activity.

Next we wanted to determine how E2A inhibits IL-22 production. Previous studies have shown that AhR-deficient ILC3s, similar to ID2-deficient ILC3s, show reduced IL-7Rα and IL-23R expression (Qiu et al., 2012). In addition, AhR is a member of the bHLH transcription factor family. Although there was a slight reduction of AhR expression in ID2-deficient ILC3s, we also considered the possibility that E2A could interact with AhR to prevent its transcriptional activity and thus result in a reduction of IL-22 in ID2-deficient ILC3s. Indeed, using EL4 cells expressing the double-FLAG-epitope-tagged (DFTC) AhR with or without E47, we detected an interaction between AhR and E2A by coimmunoprecipitation (Figure 6G). Furthermore, a chromatin immunoprecipitation (ChIP) assay revealed that E2A could suppress AhR binding to *Il22* in EL4 cell lines (Figure 6H). Together, these data suggest that E2A might regulate IL-22 production by directly binding to AhR and preventing its transcription activity at *Il22*.

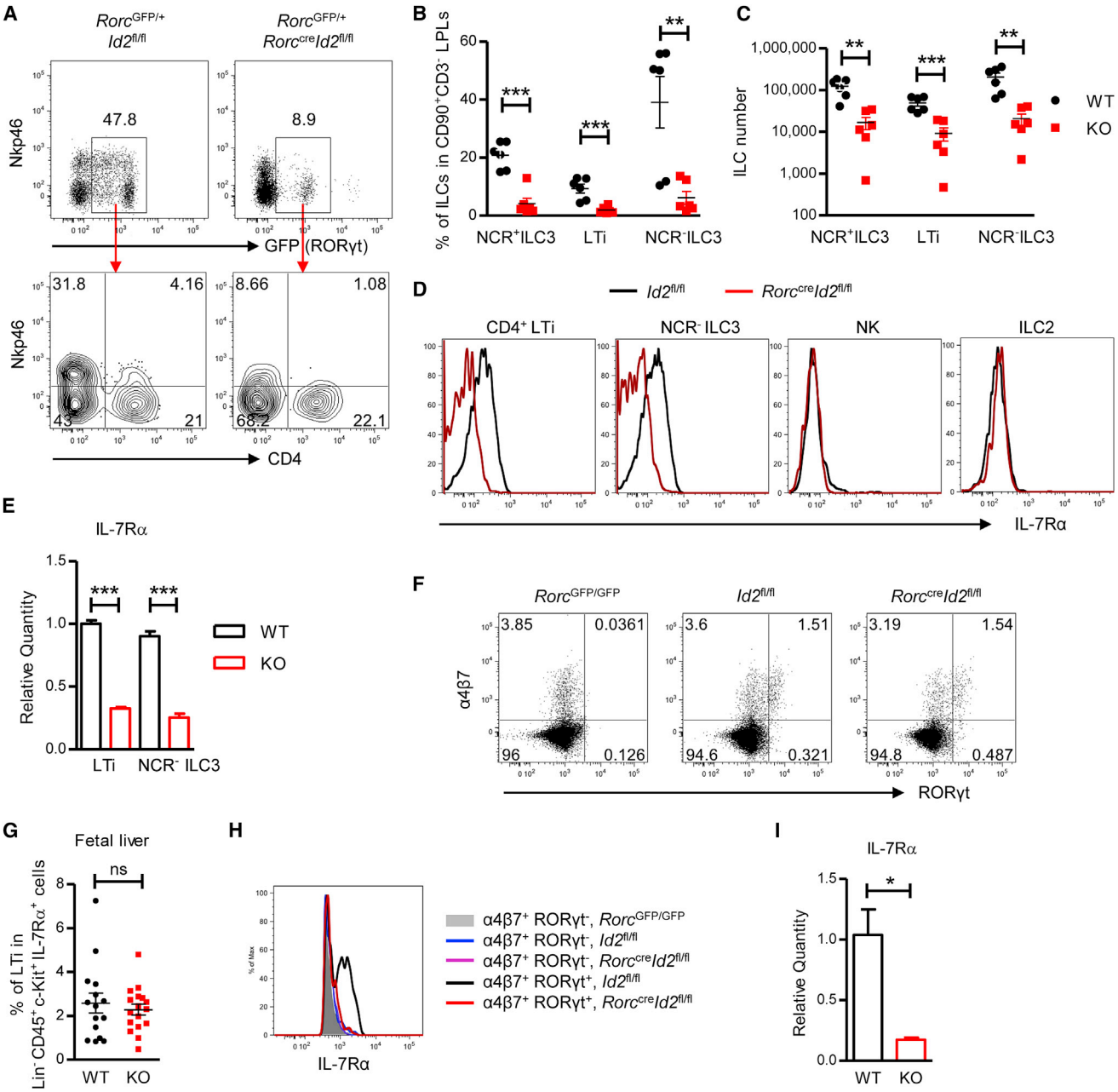

Figure 5. Continued Expression of ID2 Is Required for Further Development and Maintenance of ILC3s

(A and D) Small intestinal LPLs were isolated from 4-week-old *Rorc*$^{GFP/+}$*Id2*$^{fl/fl}$ (WT) and *Rorc*$^{GFP/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice and gated in live CD90$^+$CD3$^-$ ILCs. (A) Different populations of ILCs were analyzed by flow cytometry. Numbers adjacent to outlined areas indicate the percentage of cells in each gate. (D) The expression of IL-7Rα on different ILCs was analyzed by flow cytometry. Data are representative of three independent experiments. ILCs and their corresponding markers are as follows: NCR$^+$ ILC3s, Nkp46$^+$CD4$^-$RORγt$^+$ ILCs; LTi cells, Nkp46$^-$CD4$^+$RORγt$^+$ ILCs; NCR$^-$ ILC3s, Nkp46$^-$CD4$^-$RORγt$^+$ ILCs; NK cells, Nkp46$^+$RORγt$^-$ ILCs; and ILC2s, RORγt$^-$Nkp46$^-$ ILCs.

(B and C) The percentage of different ILC3s in the CD90$^+$CD3$^-$ ILCs and the absolute numbers of RORγt$^+$ ILC3s in the small intestine of *Id2*$^{fl/fl}$ (WT) and *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice are shown. Each dot represents one individual mouse. Data are pooled from two independent experiments (mean ± SEM).

(E) Small intestinal LPLs were isolated from 4-week-old *Rorc*$^{GFP/+}$*Id2*$^{fl/fl}$ (WT) and *Rorc*$^{GFP/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice. LTi cells and NCR$^-$ ILC3s were purified by flow cytometric sorting. The mRNA expression of IL-7Rα was measured by real-time PCR. Data are representative of two independent experiments (mean ± SEM of triplicate samples of real-time PCR).

(F and G) LTi cells in fetal liver were analyzed by flow cytometry. Embryonic day 14 and 15 fetuses were isolated and genotyped by PCR. (F) Fetal liver cells were isolated from *Rorc*$^{GFP/GFP}$, *Id2*$^{fl/fl}$, and *Rorc*^{cre}*Id2*$^{fl/fl}$ fetuses and gated in Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^+$ ILCs. Numbers adjacent to outlined areas indicate the percentage of cells in each gate. Data are representative of four independent experiments. (G) The percentage of LTi cells (α4β7$^+$RORγt$^+$) in the Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^+$ cells from (F) is shown. Each dot represents one individual mouse. Data are pooled from four independent experiments (mean ± SEM).

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DISCUSSION

Host sequential responses from innate and adaptive immune cells are essential for late-stage colonization resistance and clearance of pathogens. Here, our study demonstrates that the microbiota is important for early colonization resistance ahead of innate and adaptive responses. Fecal microbiota transplantation has been successfully used in some patients with *Clostridium difficile* infection (Austin et al., 2014). However, because the stability of the gut microbiota is dependent on many host and environmental factors (Lozupone et al., 2012), it remains unclear how a stable healthy microbial community should be introduced to prevent recurrent infection. Our results further demonstrate that baseline IL-22 production by ILC3s can regulate gut microbial homeostasis to promote colonization resistance against pathogens. Therefore, innate immunity contributes to colonization resistance not only by rapidly responding after invasion but also by maintaining the proper microbiota to limit colonization before invasion. Our study suggests that a combination of microbiota transplantation and immune molecule treatment might restore a stable microbial community to prevent intestinal pathogen colonization.

Commensals have been shown to utilize several different mechanisms to directly mediate colonization resistance against pathogens, including competing for niches and nutrients, altering host environmental conditions (for example, pH), producing bacteriocins, and affecting pathogen virulence by O_2 consumption and production of specific metabolites such as the short-chain fatty acid (Kamada et al., 2013). Previous studies have shown that *E. coli* can directly compete with *C. rodentium* for nutritional resources and help the host to clear the pathogen (Kamada et al., 2012). Repopulation of SFBs partially protects Jackson B6 mice from *C. rodentium* infection (Ivanov et al., 2009). However, we detected very few *E. coli* in the mice housed in our specific-pathogen-free facility, and the SFB level was greatly increased in conditional ID2-deficient mice, consistent with other IL-22 deficient mice (Qiu et al., 2013; Upadhyay et al., 2012). Thus, IL-22-dependent colonization resistance is not mediated through *E. coli* and SFB. It remains to be determined which commensals are regulated by innate IL-22 for colonization resistance and how commensals suppress *C. rodentium* colonization, which might lead to discovery of novel therapeutic probiotics and prebiotics.

Notch, ID2, and RORγt sequentially orchestrate the development of ILC3s (Cherrier et al., 2012). However, the function of the highly expressed ID2 in differentiated ILCs is unclear. Our data clearly demonstrate that the maintenance of ID2 expression is required for further development and function of ILC3s. Consistent with the role of ID2 on IL-7Rα regulation in $CD4^+$ thymocytes (Jones-Mason et al., 2012), we found that ID2 controls IL-7Rα expression in ILC3s, but how ID2 regulates IL-7Rα remains to be determined. Given that IL-7R signaling is also essential for the homeostasis of the other ILCs (Hoyler et al., 2012), our results suggest that ID2 might globally and constantly regulate the ILC lineage from progenitor to effector ILCs.

After IL-23 stimulation, STAT3, AhR, and RORγt can be recruited to *Il22* to promote IL-22 production. It has been shown that AhR requires interaction with RORγt to bind to *Il22*. Without RORγt, there is only a little recruitment of AhR to *Il22* (Guo et al., 2014; Qiu et al., 2012). Consistently, our data confirm that AhR weakly binds to *Il22* without overexpressing RORγt. However, E2A physically interacted with AhR and completely blocked the binding of AhR to *Il22*. Our studies suggest that two bHLH transcription factors, E2A and AhR, might form a heterodimer that either fails to bind DNA or has a DNA-binding specificity different from that of the AhR-RORγt dimer. Therefore, an essential role of ID2 might be to liberate AhR from E2A and allow AhR to bind to other transcriptional activators, such as RORγt. Whether E2A also interrupts the interaction between AhR and RORγt remains to be examined. We also showed that ID2 is required for expression of IL-7Rα and IL-23R. Given that AhR-deficient ILC3s also exhibit impaired expression of these two cytokine receptors (Qiu et al., 2012), E2A might also suppress their expression through its interaction with AhR; however, an complete understanding of the molecular mechanism by which E2A affects gene expression in ILC3s requires further investigation.

Taken together, our studies demonstrate several important findings: (1) ID2 is essential not only for ILC lineage specification but also for the maintenance and further development of $RORγt^+$ ILC3s; (2) ID2-E2A interaction regulates the function of IL-22 production in ILC3s through the AhR and IL-23R pathway; and (3) IL-22-producing ILC3s are essential for the maintenance of the proper microbiota to mediate early colonization resistance against pathogens. Proper addition of immune molecule treatment to fecal microbiota transplantation might help the host to reestablish a more stable and healthier microbial community in patients with recurrent gut infection.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and $Rag1^{-/-}$ mice were purchased from Harland Teklad. $Rag2^{-/-}Il2rg^{-/-}$, $Rorc^{GFP/+}$ (Eberl et al., 2004), and $Id2^{GFP/+}$ (Rawlins et al., 2009) mice were purchased from The Jackson Laboratory. Crossing $Id2^{fl/fl}$ mice (Niola et al., 2012) with $Rorc^{cre}$ transgenic mice (Eberl and Littman, 2004) generated $Rorc^{cre}Id2^{fl/fl}$ mice. All mice were on a C57BL/6 background. Germ-free $Ltbr^{-/-}$ mice were rederived in Taconic. All germ-free mice were maintained in the gnotobiotic facility at The University of Chicago. Animal care and use were in accordance with institutional and NIH guidelines, and all studies were approved by the animal care and use committee of The University of Chicago.

Infection with *C. rodentium* and Treatment

Mice were orally gavaged with *C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection), and body weight, survival, CFU counts, tissue histology, and PAS staining were assessed as previously described (Tumanov et al., 2011; Wang et al., 2010). Where indicated, mice were treated with antibiotics (1 g/l ampicillin, 1 g/l neomycin, 1 g/l metronidazole, and 0.5

(H) The expression of IL-7Rα on different fetal liver cells was analysis by flow cytometry. The fetal liver cells were gated in $Lin^-CD45^+c\text{-}Kit^+IL\text{-}7Rα^+$ cells first and then gated in either $α4β7^+RORγt^+$ LTi cells or $α4β7^+RORγt^-$ LTi precursor cells. Data are representative of four independent experiments.
(I) Fetal LTi cells were purified by flow cytometric sorting from fetal liver cells of $Rorc^{GFP/+}Id2^{fl/fl}$ and $Rorc^{GFP/+}Rorc^{cre}Id2^{fl/fl}$ mice. The mRNA expression of IL-7Rα was measured by real-time PCR. Data are representative of two independent experiments (mean ± SEM of triplicate samples of real-time PCR).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, no significant difference (Student's t test). See also Figure S5.

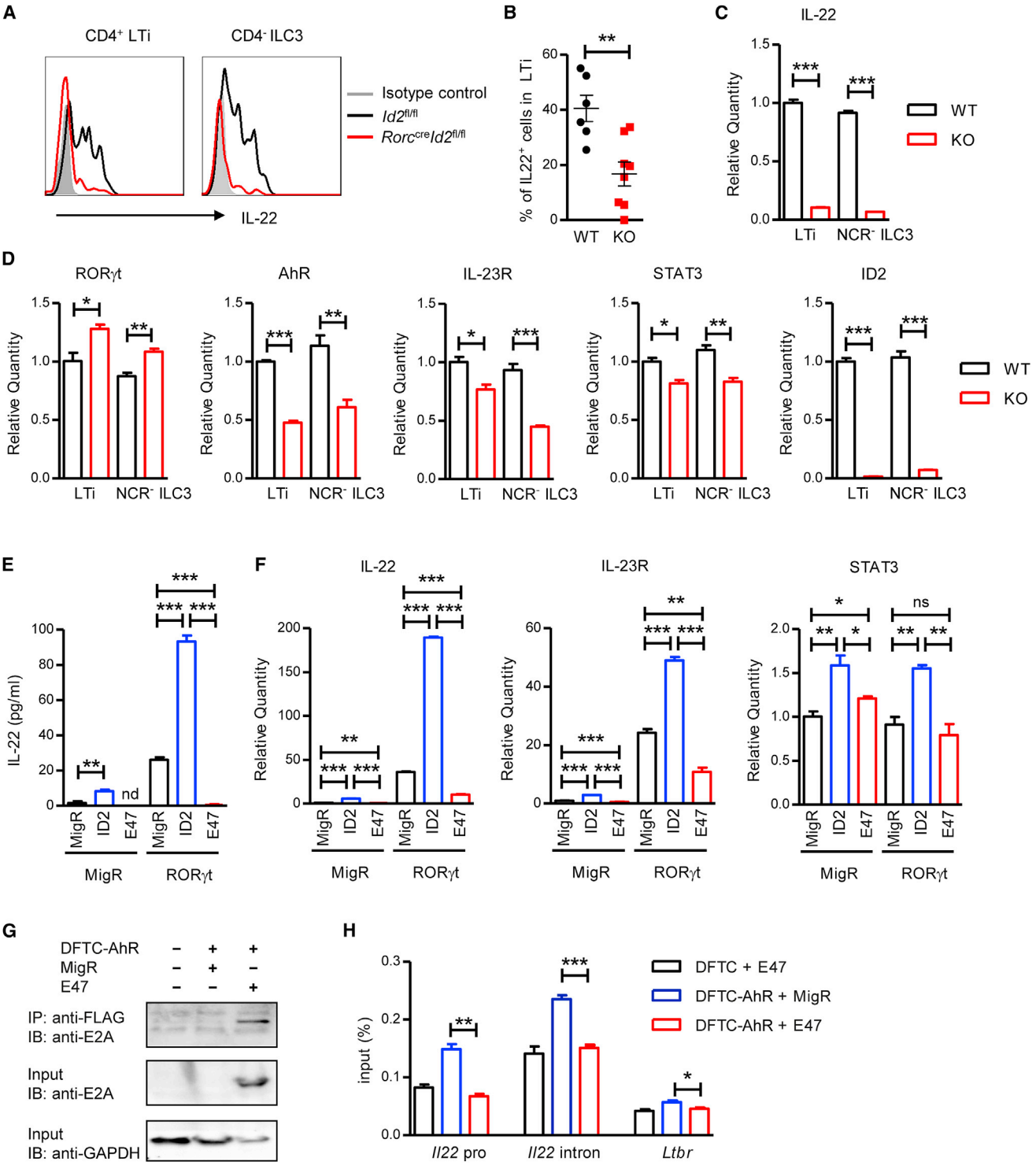

Figure 6. ID2 Regulates IL-22 Production by ILC3s through the IL-23R Pathway

(A) Intestinal LPLs were isolated from naive *Id2*$^{fl/fl}$ (WT) or *Rorc*^{cre}*Id2*$^{fl/fl}$ (KO) mice and stimulated with IL-23 (25 ng/ml) for 4 hr. IL-22 expression in CD3$^-$CD90^{hi}CD45^{lo}CD4$^+$ LTi cells or CD3$^-$CD90^{hi}CD45^{lo}CD4$^-$ ILC3s was analyzed by intracellular cytokine staining followed by flow cytometry. Data are representative of three independent experiments.

(B) Percentages of IL-22-producing cells in LTi cells are shown. Colonic LPLs were isolated from *C. rodentium*-infected WT or KO mice at post-infection day 5, restimulated with IL-23, and gated in CD3$^-$CD90^{hi}CD45^{lo}CD4$^+$ LTi cells. Each dot represents one individual mouse. Data were pooled from two independent experiments (mean ± SEM).

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g/l vancomycin) in drinking water for 1 week and then infected with *C. rodentium* after 1 day's rest. Where indicated, mice were injected intraperitoneally with either anti-IL-22 antibody (8E11.9) or mouse immunoglobulin G1 (IgG1) as an isotype control (100 μg per mouse) by 3, 4, or 5 weeks of age or on the indicated day. Then the mice were infected with *C. rodentium* at 7 weeks of age. For depletion of ILCs, *Rag1*$^{-/-}$ mice were injected intraperitoneally with anti-CD90 antibody (30H12, 100 μg per mouse) or rat IgG 10 days before *C. rodentium* infection. Where indicated, innate cells were isolated from the intestine of *Rag1*$^{-/-}$ mice and transferred by intravenous injection (1×10^6 innate cells or 2×10^5 purified CD45^{lo}CD90^{hi} ILC3s per mouse) into 3- to 4-week-old *Rorc*^{cre}*Id2*$^{fl/fl}$ mice.

Germ-Free Experiments

C57BL/6 or *Ltbr*$^{-/-}$ germ-free mice were transferred to a specific-pathogen-free environment and immediately gavaged with fresh cecal contents from *Id2*$^{fl/fl}$ and *Rorc*^{cre}*Id2*$^{fl/fl}$ littermate donors or mice treated with anti-IL-22 antibody or an isotype control. One day later, these microflora-reconstituted mice were infected with *C. rodentium* (Ahern et al., 2014).

Isolation of Intestinal LPLs and Fetal Liver Cells

The intestinal LPLs were isolated by the Lamina Propria Dissociation Kit (Miltenyl Biotec) according to the manufacturer's recommendations. Fetal liver cells were dissociated by mechanical shearing in PBS containing 0.5% bovine serum and then filtered through a 70-μM mesh.

Flow Cytometry, Antibodies, and ELISA

Antibodies against a lineage marker, CD3, CD4, CD45, CD90, Nkp46, CD117, CD127, α4β7, RORγt, and streptavidin-APC were purchased from BioLegend or eBioscience. Anti-IL-22 antibody was a gift from Genentech. For nuclear staining, cells were fixed and permeabilized with the Transcription Factor Staining Buffer Set (eBioscience). For cytokine production, cells were stimulated ex vivo by IL-23 (25 ng/ml; R&D Systems) for 4 hr. IC Fixation Buffer and Permeabilization Buffer (eBioscience) were used for intracellular cytokine staining. Flow cytometry was performed on LSR-Fortessa instruments (BD Biosciences) and analyzed with FlowJo software (Tree Star). ILC3s were sorted from the intestinal LPLs and fetal liver cells on a FACSAria III instrument (BD Biosciences). IL-22 in supernatants was measured by ELISA according to the manufacturer's (R&D Systems) recommendations.

Quantitative Real-Time RT-PCR

RNA isolation and real-time PCR were performed as previously described (Guo et al., 2014) with different primer sets (Table S1).

Retroviral Transduction of Cell Lines

The retroviruses expressing MigR, ID2, E47, and RORγt were made as previously described (Qiu et al., 2012). EL4 cells were infected with 2 ml of virus supernatant (MigR, ID2, or E47), and 48 hr later, the GFP$^+$ infected cells were sorted on the Avalon Cell Sorter (Propel Labs). The sorted EL4 cells were then infected with a retrovirus expressing either MigR or RORγt. Cell-culture supernatant and cell RNA were analyzed by ELISA and real-time PCR separately.

Co-immunoprecipitation and ChIP

EL4 cells were infected with DFTC AhR retroviral vector and selected in the presence of 7 μg/ml puromycin. EL4 cells stably expressing AhR were then infected with a retrovirus expressing either MigR or E47. The GFP$^+$ infected cells were sorted, and the whole-cell lysate supernatant was immunoprecipitated with EZview Red Anti-FLAG Affinity Gel (Sigma-Aldrich). Immunoblotting was performed with anti-E2A rabbit antibody (Thermo Scientific) and anti-GAPDH antibody (Sigma-Aldrich). ChIP assays with EL4 cells were performed as previously described (Qiu et al., 2012).

Statistical Methods

Statistical analysis was performed by a two-tailed Student's t test in the GraphPad Prism 5.0 program. Data from such experiments are presented as mean values ± SEM; $p < 0.05$ was considered significant. For survival curves, statistics were done with the log-rank (Mantel-Cox) test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.03.012.

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(C and D) LTi cells and NCR$^-$ ILC3s were purified by flow cytometric sorting from intestinal LPLs of *Rorc*$^{GFP/+}$*Id2*$^{fl/fl}$ or *Rorc*$^{GFP/+}$*Rorc*^{cre}*Id2*$^{fl/fl}$ mice and lysed directly for RNA extraction. The mRNA expression of IL-22 (C) and RORγt, AhR, IL-23R, and STAT3 (D) was measured by real-time PCR. Data are representative of two independent experiments (C and D) (mean ± SEM of triplicate samples of real-time PCR).
(E and F) EL4 cells were infected with ID2 or E47 expression or control retrovirus with or without RORγt expression retrovirus and stimulated with IL-23 for 2 days. (E) The production of IL-22 in the cell-culture supernatant was measured by ELISA. (F) The mRNA expression of IL-22, IL-23R, and STAT3 was measured by real-time PCR. Error bars represent the SEM of triplicate samples. Data are representative of two independent experiments.
(G) E2A physically interacted with AhR. EL4 cells were stably infected with the indicated expression constructs. Whole-cell extracts were immunoprecipitated with anti-FLAG beads and subsequently immunoblotted with anti-E2A antibodies. Data are representative of three independent experiments.
(H) Empty MigR or E47 was coexpressed by retroviral transduction in EL4 cell lines stably expressing either DFTC or DFTC-AhR. AhR binding at *Il22* was detected by ChIP assay. Data are representative of two independent experiments (mean ± SEM of triplicate samples of real-time PCR).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, no significant difference (Student's t test); nd, nondetectable. See also Figure S6.

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